Albuminuria induces a proinflammatory and profibrotic response in cortical collecting ducts via the 24p3 receptor

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Abstract

Albuminuria is strongly associated with progressive kidney tubulo-interstitial damage and chronic kidney disease (CKD) progression. In proteinuric nephropathies, albumin reabsorption by the proximal tubule is saturated and the distal nephron is exposed to high concentrations of luminal albumin that may produce adverse effects. Since proximal tubular cells exposed to albuminuria exhibit a proinflammatory and profibrotic response, we assessed the effect of albuminuria in the collecting duct (CD). With the use of kidney sections and isolated cortical CDs (CCDs) from puromycin-aminonucleoside-induced nephrotic rats (PAN rats) exhibiting proteinuria, immunofluorescence microscopy revealed internalized albumin in CD cells. In these proteinuric rats, increased expression levels of cytokines and profibrotic signaling markers were detected in isolated CCDs and bands of inflammatory fibrosis could be observed around CDs. Albumin endocytosis was confirmed by FITC-albumin uptake in cultured murine CCD (mCCDcl1) cells. Exposure of mCCDcl1 cells to albumin induced NF-κB activation as assessed by luciferase reporter gene assay, nuclear […]

Reference


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Albuminuria induces a proinflammatory and profibrotic response in cortical collecting ducts via the 24p3 receptor

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Am J Physiol Renal Physiol 305: F1053–F1063, 2013. First published July 14, 2013; doi:10.1152/ajprenal.00006.2013.— Albuminuria is strongly associated with progressive kidney tubulointerstitial damage and chronic kidney disease (CKD) progression. In proteinuric nephropathies, albumin reabsorption by the proximal tubule is saturated and the distal nephron is exposed to high concentrations of luminal albumin that may produce adverse effects. Since proximal tubular cells exposed to albuminuria exhibit a proinflammatory and profibrotic response, we assessed the effect of albuminuria in the collecting duct (CD). With the use of kidney sections and isolated cortical CDs (CCDs) from puromycin-aminonucleoside-induced nephrotic rats (PAN rats) exhibiting proteinuria, immunofluorescence microscopy revealed internalized albumin in CD cells. In these proteinuric rats, increased expression levels of cytokines and profibrotic signaling markers were detected in isolated CCDs and bands of inflammatory fibrosis could be observed around CDs. Albumin endocytosis was confirmed by FITC-albumin uptake in cultured murine CCD cells. Exposure of mCCD cells to albumin induced NF-κB activation as assessed by luciferase reporter gene assay, nuclear translocation of NF-κB p65 subunit, and increased NF-κB target gene expression. Moreover, albuminuria-like condition results in transforming growth factor-β1 (TGF-β1) overexpression and the upregulation of profibrotic signaling markers such as Smad9 or vimentin via an autocrine mechanism. In mCCD cells, neutrophil gelatinase-associated lipocalin (NGAL)/lipocalin-2/24p3 receptor (24p3R) mediates albumin endocytosis as well as activation of NF-κB and TGF-β1 signaling pathways. Therefore, CD may play a key role in initiation and/or progression of inflammation and fibrosis in response to proteinuria.

proteinuria; collecting duct; 24p3R; fibrosis

Proteinuria is a pathological process defined by the presence of >300 mg protein/day in the urine. Glomerular proteinuria mainly consists of albumin and derives from functional or physical alterations of the glomerular filtration barrier. Alterations of albumin reabsorption by the proximal tubule may also play an important role in the development of microalbuminuria (5, 31, 32). Indeed, proximal tubular cells actively reabsorb luminal peptides and proteins, including albumin via the megalin-cubulin endocytic pathway (23, 24). The physiological importance of this mechanism was further highlighted by the recent discovery of cubulin polymorphisms associated with microalbuminuria and megalin polymorphism associated with loss of renal function (5, 6). The reabsorption of filtered proteins by the proximal tubule therefore provides a protein-free luminal fluid to more distal parts of the renal tubule (26).

Proteinuria is a major predictive factor of chronic kidney disease (CKD) progression in humans (25, 30, 36). The link among proteinuria, tubulo-interstitial inflammation, and fibrosis is still debated, but albumin overload might be toxic for proximal tubular cells. Indeed, severe proteinuria is associated with altered tubular cell differentiation and viability as well as interstitial inflammation in vivo (9). In addition, studies on cultured proximal tubular cells have demonstrated that extracellular albumin overload leads to proinflammatory NF-κB activation and expression of profibrotic factors such as transforming growth factor-β (TGF-β; 40)). Therefore, proximal tubular cells may participate in tubulo-interstitial inflammation and fibrosis observed in glomerular diseases (1). Under proteinuric conditions, the reabsorptive capacity of the proximal tubule is overwhelmed and epithelial cells from more distal renal tubule segments, including the collecting duct (CD), are very likely exposed to higher concentrations of luminal albumin than proximal tubules according to 90% of filtered water reabsorption at the end of the descending limb of Henle’s loop. On the other hand, the CD was recently demonstrated to have a major role in the initiation and progression of tubulointerstitial inflammation (12).

We therefore investigated the effects of luminal albumin exposure of CD cells in vivo and in vitro. We demonstrated that albumin is internalized by CD cells. In both cultured cells and CDs from proteinuric rats, albumin endocytosis activated NF-κB and TGF-β1 signaling pathways resulting in expression of proinflammatory and profibrotic signaling markers, respectively. In addition, we show that neutrophil gelatinase-associated lipocalin (NGAL)/lipocalin-2/24p3 receptor (24p3R) mediates albumin internalization in CD cells and its consequences on proinflammatory and profibrotic signals.

Methods

Antibodies. Anti-albumin FITC-conjugated (F0117) was purchased from DAKO, rabbit polyclonal anti-24p3R (SLC22A17) from Abnova, mouse monoclonal anti-β-actin (A5441) and mouse monoclonal anti-α-tubulin (T9026) from Sigma, mouse monoclonal anti-p65 (sc8008) and anti-HDAC3 (sc2632) from Santa Cruz, and rabbit polyclonal anti-phospho-Smad3 (C25A9) and anti-Smad3 (C67H9) from Cell Signaling. Rabbit polyclonal anti-aquaporin 2 (AQP2) antibodies (AB7661) were a kind gift of R. Fenton (University of Aarhus, Aarhus, Denmark).

Puromycin-aminonucleoside-induced nephrotic syndrome. All animal experiments were approved by the Institutional Ethical Committee of Animal Care in Geneva and Cantonal authorities. Male Wistar
Table 1. Biochemical data

<table>
<thead>
<tr>
<th>Data</th>
<th>Control (n = 10)</th>
<th>PAN Rats (n = 6)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na, mmol/l</td>
<td>139 ± 1</td>
<td>138 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>K, mmol/l</td>
<td>4.6 ± 0.4</td>
<td>5.4 ± 0.36</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine, µmol/l</td>
<td>23.63 ± 0.4</td>
<td>30 ± 4</td>
<td>NS</td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>2.28 ± 0.15</td>
<td>1.4 ± 0.44</td>
<td>0.03</td>
</tr>
<tr>
<td>Urine volume, ml/24 h</td>
<td>8.9 ± 0.8</td>
<td>7.6 ± 1.6</td>
<td>NS</td>
</tr>
<tr>
<td>Proteinuria/creatinine, g/mmol</td>
<td>0.04 ± 0.01</td>
<td>2.5 ± 0.3</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of rats. PAN rats, puromycin-aminonucleoside-induced nephrotic rats; GFR, glomerular filtration rate; NS, not significant.

Table 2. Sequences of primers

<table>
<thead>
<tr>
<th>Species/Targeted Gene</th>
<th>Forward 5' - 3'</th>
<th>Reverse 5' - 3'</th>
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<tr>
<td>Mouse</td>
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<td></td>
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<td>TNF-α</td>
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<tr>
<td>LacI</td>
<td>CGGAGGAGGAGACTCTTCTTT</td>
<td>TTCCAGTTGAAATGCTTCAA</td>
</tr>
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<td>RANTES</td>
<td>TCTTCTGATGTTTTGGTCA</td>
<td>GGGTGAAATGCAAAGCC</td>
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<tr>
<td>TGF-β1</td>
<td>CGGAGGAGCTGATAGGCA</td>
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<tr>
<td>α-SMA</td>
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<td>AAAGAAAAGCCAGTAAACAACTC</td>
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<td>Vimentin</td>
<td>CGGTTCAGAGACAGCAAG</td>
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<td>BMP-7</td>
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<td>24p3R</td>
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<td>GACGGATTGAAGAAAGGAGCA</td>
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<td>Rat</td>
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<td>MCP-1</td>
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<td>IL-1β</td>
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<td>TGF-β1</td>
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<td>α-SMA</td>
<td>AATGCTGAGGATGCTTTCCT</td>
<td>TTTCTGACGAGAGAGAGGAG</td>
</tr>
<tr>
<td>Vimentin</td>
<td>CGAGGAGACAGTTGGAGAGGAG</td>
<td>GGCTGGGATTGGAGAGAGGAG</td>
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<tr>
<td>24p3R</td>
<td>GGAGAGCGCGGAGCAAGAGGAG</td>
<td>GGGCTGAGAGGAGAGGAG</td>
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</tbody>
</table>

RANTES, regulated on activation normal T-expressed and presumably secreted; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; BMP-7, bone morphogenic protein-7; 24p3R, 24p3 receptor; MCP-1, monocyte chemoattractant protein-1.
collected (cytoplasmic fraction) and the pellet was resuspended in a hypertonic buffer (2 mM HEPES pH 8, 450 mM KCl, 1.5 mM MgCl₂, and 25% glycerol) supplemented with protease inhibitors. After incubation for 30 min at 4°C, the supernatant (nuclear fraction) was collected by centrifugation (13,000 rpm, 5 min). Protein concentrations were determined by the Bradford procedure (Bio-Rad). Proteins were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) using standard methods.

Luciferase assay. Cells were cotransfected with (pB)3 IFN-Luc plasmid encoding Firefly luciferase under the control of three kB-enhancer elements (11) and pRL vector (Promega, Madison, WI) encoding Renilla luciferase used as control. Luciferase activity was measured using the Dual Luciferase Assay System (Promega) according to the manufacturer’s instructions. The light produced was measured using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

Fig. 1. Albumin is internalized by collecting duct cells. A: immunolabeling of kidney sections from control or puromycin-aminonucleoside-induced nephrotic rats (PAN) using anti-albumin (green) and anti-aquaporin 2 (AQP2; red) antibodies. *Proximal tubules; **glomeruli. B: immunolabeling of microdissected cortical collecting ducts with anti-anion exchanger AE1 (red) and anti-albumin (green) antibodies. C: confocal microscopy analysis of murine cortical collecting duct (mCCDcl1) cells exposed to FITC-labeled albumin (green) and LysoTracker Red for 30 min at 37°C. D: dose response (0.001 to 0.1 mg/ml) of albumin-FITC uptake by mCCDcl1 cells measured by flow cytometry. E: time course (0.5 to 24 h) of albumin-FITC (0.01 mg/ml) uptake by mCCDcl1 cells measured by flow cytometry (a.u., arbitrary units).
**Statistics.** Results are given as the means ± SE from \( n \) independent experiments. Comparisons between two groups were performed by unpaired Student’s t-test if not stated otherwise.

**RESULTS**

**Apical albumin is internalized by CD cells.** Immunofluorescence analysis of albumin on kidney sections from puromycin-aminonucleoside (PAN) nephrotic rats with proteinuria revealed strong albumin staining of proximal tubules but also of AQP2-positive tubules [CCDs and connecting tubules (CNTs); Fig. 1A]. AQP2-positive tubules were not uniformly labeled, as expected in this model of focal glomerular disease. Intracellular albumin was observed in both principal (AQP2 positive) and intercalated (AQP2 negative) cells. Albumin internalization by CD cells was further confirmed using microdissected cortical CDs (CCD) from PAN or control rats. Intracellular albumin labeling was observed in both principal and intercalated cells (counterstained for AE1) from CCDs of PAN rats (Fig. 1B). Confocal microscopy analysis of cultured mCCDcl1 principal cells exposed to apical FITC-labeled albumin and Lyso Tracker Red, a marker of late endosomes and lysosomes, revealed a strong intracellular fluorescence corresponding to albumin internalization and degradation in lysosomes (Fig. 1C). This internalization was dose dependent as confirmed by measurement of FITC-albumin intake by flow cytometry (Fig. 1D). Time-course experiments of albumin uptake in mCCDcl1 cells revealed that this process is saturable, suggesting that albumin uptake is a receptor-mediated process in CD cells (Fig. 1E).

**Proinflammatory and profibrotic signaling markers are upregulated in CDs of proteinuric rats.** Albuminuria is strongly correlated with CKD progression and therefore renal tubulointerstitial fibrosis (4, 13). Since we observed albumin endocytosis in CDs of proteinuric rats, we analyzed the expression of proinflammatory and profibrotic signaling markers in these animals. Real-time PCR analysis of renal cortex and microdissected CDs revealed higher regulated on activation normal T-expressed and presumably secreted (RANTES), α-smooth muscle actin (α-SMA), and vimentin mRNA levels in PAN rats compared with control rats (Fig. 2A) both 7 and 14 days after PAN injection. Microdissected CD purity was assessed by detection of high AQP2 mRNA expression levels while NaPi-2a, a proximal tubule marker, was not expressed (Fig. 2B). AQP2 mRNA expression was lower in PAN treated rats. Imaging of collagen accumulation by unpolarized Sirius red staining of kidney sections from PAN or control rats revealed areas of interstitial fibrosis in cortical labyrinths in PAN rats (Fig. 2C, middle, arrow), as expected. Fibrosis was also detected along medullary rays and surrounding CCDs (Fig. 2C, right, arrows), identified by AQP2 immunohistochemistry on kidney serial sections.

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**Fig. 2.** Proinflammatory and profibrotic signaling markers are upregulated in collecting ducts of nephrotic rats. A: relative mRNA levels of proinflammatory and profibrotic signaling marker genes were measured in kidney cortex or microdissected CCD of PAN nephrotic or control rats 7 or 14 days after PAN or vehicle injection. B: purity of microdissected tubules was controlled by real-time PCR analysis of NaPi-2a and AQP2 mRNA abundance in microdissected proximal convoluted tubules (PCT) and CCD. C: unpolarized Sirius red staining that labels fibrillar collagen and AQP2 immunohistochemical staining that labels connecting tubules and collecting ducts were performed on serial kidney sections from PAN nephrotic and control rats. Two representative images are shown.
Albuminuria induces a proinflammatory response via NF-κB activation. Because albuminuria induces a proinflammatory response in proximal tubular cells (39), we investigated albumin’s effect on the major proinflammatory NF-κB pathway in cultured CD principal cells. We first tested whether albumin modulates NF-κB transcriptional activity in mCCD_{11} cells transiently transfected with a luciferase reporter gene under the control of NF-κB elements. In cells exposed to endotoxin- and lipid-free albumin, luciferase activity increased in a dose-dependent manner with a threshold effect observed at 10 mg/ml (Fig. 3A). This concentration was used to show the nuclear translocation of NF-κB complex p65 subunit observed after 30-min albumin exposure (Fig. 3B). In addition, mRNA levels of three well-described NF-κB target genes increased in a dose-dependent manner in mCCD_{11} cells exposed to albumin for 3 h (Fig. 3C). The activation of NF-κB is often described as a transient process (37). We analyzed kinetics of NF-κB activation in mCCD_{11} cells exposed to albuminuria-like conditions for 3, 6, and 24 h. The expression levels of TNF-α IκBα and RANTES mRNA started to increase 3 h after albumin exposure but clearly decreased after 24 h, reflecting a transient activation of NF-κB pathway (Fig. 3D). We next investigated whether high concentrations of albumin could modify osmolarity, resulting in NF-κB activation via the osmoprotective transcription factor TonEBP (29). Addition of albumin up to 25 mg/ml did not modify the measured osmolarity of the incubation medium. In addition, the exposure of mCCD_{11} cells to 10 mg/ml albumin for 6 h did not alter the expression levels of aldose reductase, a typical TonEBP-responsive gene (data not shown). Finally, TonEBP silencing by siRNA did not prevent the increase in TNF-α expression in response to albumin (data not shown). Therefore, NF-κB pathway activation by albumin is independent of TonEBP activity.

Fig. 3. Albuminuria activates the NF-κB pathway in collecting duct cells. A: mCCD_{11} cells were co-transfected with firefly luciferase under the control of specific NF-κB elements together with renilla luciferase under the control of a constitutive promoter. Cells were treated for 6 h with 10 ng/ml LPS or increasing concentrations (0.1–20 mg/ml) of albumin before measurement of luciferase activity. Results were calculated as a ratio of firefly/renilla luciferase activity and expressed as fold of control (Ctl) values. Results are expressed as means ± SE from 6 independent experiments. B: cytosolic and nuclear fractions of mCCD_{11} cells exposed to 10 mg/ml albumin for 10, 30, and 60 min were analyzed by Western blot using anti-p65, anti-HDAC3, and anti-α-tubulin antibodies. HDAC3 and α-tubulin were used as nuclear and cytosolic markers respectively. C and D: relative mRNA levels of TNF-α, IκBα, and regulated on activation normal T-expressed and presumably secreted (RANTES) were quantified by real-time PCR using the housekeeping gene acidic ribosomal phosphoprotein P0 in mCCD_{11} cells exposed to increasing concentrations of albumin (0.1–30 mg/ml) or to 10 mg/ml albumin for different periods of time (3, 6, and 24 h). Results are means ± SE from 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Albuminuria induces TGF-β pathway. Since interstitial fibrosis is strongly linked to CKD progression (33) and because albumin was reported to induce TGF-β production by proximal tubule cells (38), we analyzed whether albuminuria activates the TGF-β1 pathway in mCCDcl1 cells. In cells exposed to various concentrations of albumin for 3 h we observed a dose-dependent increase of TGF-β1 mRNA expression with a threshold effect at 1 mg/ml (Fig. 4A). Time-course analysis of albumin exposure revealed that TGF-β1 mRNA upregulation is maintained for at least 24 h (Fig. 4B). The biological activity of albumin-induced TGF-β1 expression was assessed by analysis of the phosphorylation levels of the transcription factor Smad3 and the expression levels of TGF-β1 target genes. Increased phosphorylation of Smad3 was detected 1 h after albumin exposure and was sustained for at least 24 h (Fig. 4C). Furthermore, TGF-β1 target genes expression increased either after 3 h (Snail), after 6 h (α-SMA), or after 48 h (vimentin), showing that activation of TGF-β1 pathway and the expression of profibrotic signaling markers in response to albuminuria-like conditions is maintained over time (Fig. 4D). In mCCDcl1 cells treated with SB431542, a specific TGF-β receptor blocker, the increases of Snail and α-SMA expression induced by albumin or TGF-β1 were abolished (Fig. 4E). Therefore, albuminuria-like conditions induce TGF-β1 upregulation and activate TGF-β1 signaling pathway through TGF-β1 and the canonical Smad-dependent pathway. These observations suggest that albuminuria-like conditions induce autocrine TGF-β1 signaling in CD cells. Using siRNAs specifically targeting p65 or/and p50 NF-κB subunits, we observed that increased TGF-β1 expression in response to albumin does not rely on NF-κB pathway activation in mCCDcl1 cells (data not shown).

NGAL/lipocalin-2/24p3R mediates albumin endocytosis and the subsequent activation of NF-κB and TGF-β1 pathways in CD cells. Albumin endocytosis by the proximal tubule is mediated by the megalin/cubulin receptor complex that is specifically expressed in this nephron segment (7). Expression of NGAL receptor or 24p3R has been recently described in distal tubules and CDs of mouse kidney where it could mediate protein endocytosis, especially albumin (18). Immunohistochemistry analysis of serial sections revealed that 24p3R is expressed in cortical AQP2-positive tubules corresponding to both CNTs and CCDs (Fig. 5A). Both principal and intercalated cells displayed apical staining for this receptor. In addition, some AQP2-negative tubules [distal tubules as already

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

Fig. 4. Albuminuria induces transforming growth factor-β1 (TGF-β1) expression and signaling in mCCDcl1 cells. A and B: relative TGF-β1 mRNA levels were quantified by real-time PCR in mCCDcl1 cells exposed to increasing concentrations of albumin (0.1 to 30 mg/ml; A) or to 10 mg/ml albumin for different periods of time (3, 6, and 24 h; B). C: Western blot analysis of Smad3 phosphorylation kinetics in mCCDcl1 cells exposed to 10 mg/ml albumin. D: quantification of relative mRNA levels of profibrotic signaling markers Snail, α-smooth muscle actin (α-SMA), and vimentin in mCCDcl1 cells treated with albumin for 3 to 72 h (D) or in mCCDcl1 cells exposed to albumin or TGF-β1 for 3 h and cotreated or not with TGF-β1 receptor kinase inhibitor SB4321542 (I) (E). Results are means ± SE from 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

AJP-Renal Physiol doi:10.1152/ajprenal.00006.2013 www.ajprenal.org
described (18] also expressed 24p3R (data not shown). Finally, inner medullary collecting ducts also displayed 24p3R staining (Fig. 5A). To determine the role of this receptor in albumin endocytosis, we quantified FITC-albumin uptake in mCCDcl1 cells after 24p3R knockdown. Transfection of siRNA targeting 24p3R decreased 24p3R mRNA and protein expression by \( \frac{1}{2} \times 100 \% \), as observed by real-time PCR and Western blot, respectively (Fig. 5B). Measurement of FITC-albumin uptake by flow cytometry in mCCDcl1 cells revealed that 24p3R silencing reduced albumin uptake by \( \frac{1}{2} \times 100 \% \), indicating

Fig. 5. 24p3R receptor mediates albumin endocytosis and activation of NF-κB and TGF-β signaling pathways. A: 24p3R and AQP2 immunohistochemical staining was performed on serial kidney sections of control rats. *CCDs. B: analysis of 24p3R mRNA levels by real-time PCR (left) or protein abundance by Western blotting (right) in mCCDcl1 cells transfected with siRNA targeting 24p3R (si24p3R) or control scramble RNA (Ctl). C: flow cytometry analysis of FITC-labeled albumin uptake in mCCDcl1 cells transfected with si24p3R or control siRNA and exposed or not to 0.01mg/ml albumin-FITC. Cells exposed to albumin-FITC but maintained at 4°C to block endocytosis were used as a negative control. D–F: relative mRNA levels of TNF-α, IκBα and RANTES (D), TGF-β (E), Snail, and α-SMA (F) were quantified by real-time PCR in mCCDcl1 cells transfected with si24p3R or control siRNA (Ctl) and exposed to 10 mg/ml albumin for different periods of time (3, 6, and 24 h). Results are means ± SE from 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
that 24p3R significantly contributes to albumin endocytosis in mCCDc11 cells (Fig. 5C). Since albuminuria-like conditions increased the mRNA levels of NF-κB-induced genes, we assessed the effect of albumin on NF-κB target genes expression after 24p3R silencing in mCCDc11 cells. As shown in Fig. 5D, upregulation of NF-κB target genes such as TNF-α, IkBα, and RANTES in response to albuminuria-like conditions was blunted in cells depleted for 24p3R compared with control cells. The same experimental conditions were used to analyze the effect of 24p3R inhibition on TGF-β1, and profibrotic signaling markers expression levels. The upregulation of TGF-β1, Snail, and α-SMA mRNA was almost abolished in 24p3R-depleted cells (Fig. 5, D–F). Thus 24p3R mediates both NF-κB and TGF-β1 pathways activation through albumin internalization in mCCDc11 cells.

**Downregulation of 24p3R in response to albuminuria.** Exposure of mCCDc11 cells to albumin for 3–48 h revealed a downregulation of both 24p3R mRNA and protein (Fig. 6, A and B). This observation was confirmed by real-time PCR analysis of microdissected CDs from PAN rats compared with control animals (Fig. 6C), whereas 24p3R was still clearly detected by immunohistochemistry in CDs of PAN rats (Fig. 6D). To study whether TGF-β1 plays a role in this mechanism, mCCDc11 cells were treated with TGF-β1 for 12 or 24 h. That treatment clearly induces a decrease of 24p3R expression similar to albuminuria-like conditions (Fig. 6, E and F), suggesting the involvement of TGF-β1 in the 24p3R downregulation induced by albumin.

**DISCUSSION**

We provide experimental evidence in vivo and in vitro that luminal albumin is not exclusively handled by proximal tubular cells but also by CD cells (Fig. 7). We further demonstrate for the first time that internalization of albumin in the CD is partially dependent on the 24p3R and induces a proinflammatory and profibrotic autocrine response in cultured mCCDc11 cells as well as in CDs from proteinuric rats. The distal nephron may therefore participate to kidney fibrosis progression in proteinuric nephropathies.

The link between albuminuria and CKD progression is well established by epidemiological studies (4, 20, 21). Albumin toxicity is well described in the proximal tubule and appears related to albumin endocytosis (1). However, appearance of albumin in patient’s urine implies saturation of the proximal tubule endocytotic mechanisms. Therefore, in the presence of albuminuria, distal nephron segments are exposed to albumin. Furthermore, defective uptake of albumin in the proximal tubule appears to be an important mechanism contributing to albuminuria in the general and diabetic population (2, 5, 31, 32) and may also contribute to glomerular filtration rate decline (6). The effect of albumin exposure of distal part of the nephron has never been studied and may explain at least in part the correlation between albuminuria and CKD progression.

Recent experimental pieces of evidence point to the fact that tubular cells play mostly a paracrine role in kidney fibrosis (16,
Proximal as well as CD cells are indeed able to secrete both proinflammatory and profibrotic cytokines, attracting inflammatory cells and activating surrounding fibroblasts (12, 22, 34, 35). A recent study indicates that CD cells specifically play a crucial role in the initiation of tubulointerstitial inflammation and injury in the unilateral ureteral obstruction model (12).

We here show for the first time in vivo and in vitro that albumin uptake by CD cells activates the canonical NF-κB pathway leading to expression of proinflammatory cytokines. Among these, transcription of RANTES, recently demonstrated to play a major role in chemotactism by kidney epithelial cells during mouse nephropathies (22), was clearly induced in vivo in CD cells of proteinuric rats. This observation is therefore consistent with a significant role of the CD in chemotactism and inflammation initiation under proteinuric conditions. The observed downregulation of AQP2 is consistent with previous reports in PAN induced nephrotic syndrome (3). The cause of this downregulation is currently unknown. It could be related to a direct effect of proteinuria, or of proinflammatory factors, since proinflammatory factors may downregulate AQP2 expression (14, 28).

We further show in vivo that mRNA expression of vimentin and α-SMA are induced in CCDs of proteinuric rats at 7 and 14 days, implying local activation of the TGF-β1 pathway. This was seen together with the apparition of fibrosis bands not only in cortical labyrinths, as previously described, but also in some cases in the vicinity of CDs in medullary rays. Albuminuria-induced TGF-β1 expression in cultured CD cells, together with an increased expression of its target genes such as Snail and α-SMA that is prevented by blockade of TGF-β receptor 1, is suggestive of an autocrine mechanism. The CD may therefore participate in the activation of surrounding cells and fibrosis initiation in proteinuric kidney diseases.

Megalin and cubulin are not expressed in the distal nephron. We demonstrate that 24p3R, a NGAL-/lipocalin-2 receptor, shown to mediate protein endocytosis in the distal nephron (18), is expressed in CNTs and CCDs of rats in addition to distal tubules and inner medullary collecting ducts (18). In vitro knockdown of this receptor significantly decreased albumin uptake as well as the profibrotic and proinflammatory effects of albumin exposure. This indicates that internalization of albumin via the 24p3R plays a crucial role in the observed proinflammatory and profibrotic response of the CD. Albumin internalization was not fully abolished by 24p3R silencing, in agreement with residual expression of this receptor. However, we cannot exclude that other receptors may also participate in albumin endocytosis in CD cells.

We further observed that 24p3R is downregulated in the presence of albumin in vitro and at the mRNA level in vivo, suggesting that a negative feedback loop may decrease albumin internalization over time. Despite this downregulation, 24p3R protein was still expressed in AQP2-positive tubules (CNT and CCD) in proteinuric PAN rats and probably accounts for sustained, albeit lowered, albumin uptake.

In conclusion, we demonstrate that the distal nephron is exposed to and takes up albumin under conditions of proteinuria. Albumin internalization is in large part mediated through 24p3R receptor in this segment. It induces profibrotic and proinflammatory responses characterized by autocrine secretion of TGF-β1 leading to target genes expression and by
upregulation of proinflammatory chemotactic factors production both in vitro and in vivo. Within the same time frame, bands of fibrosis are observed in the kidney cortex of proteinuric rats. Albumin endocytosis by the proximal tubule may therefore not be the single factor leading to renal tubulointerstitial fibrosis and inflammation. We postulate that the more distal segments of the kidney tubule, such as the CD, also play a role in the initiation and progression of these processes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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